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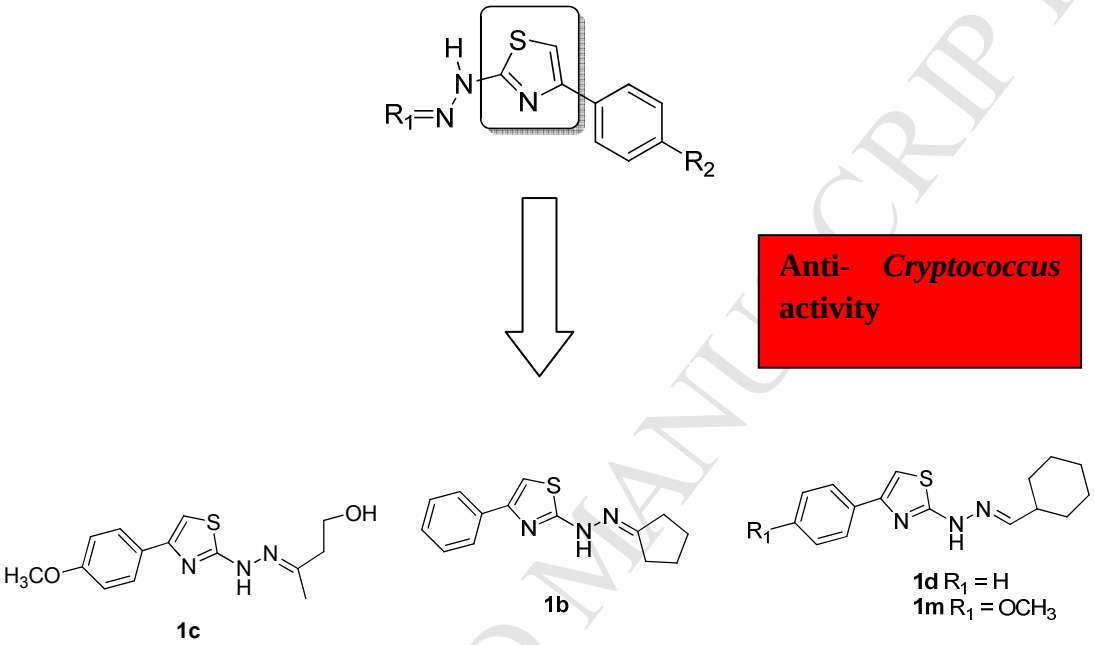
Graphical Abstract



MIC $_{90}$
C. neoformans 0.9 µM
C. gattii 1.4 µM

MIC $_{90}$
C. neoformans 3.2 µM
C. gattii 3.1 µM

MIC $_{90}$ **1d**
C. neoformans 2.2 µM
C. gattii 1.9 µM

1m
C. neoformans 3.5 µM
C. gattii 4.2 µM

Thiazole compounds with activity against *Cryptococcus gattii* and *C. neoformans in vitro*

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ABSTRACT

Human cryptococcosis can occur as a primary or opportunistic infection and develop as an acute, subacute, or chronic, systemic infection involving different host organs. We evaluated the antifungal activity of thirteen compounds against *Cryptococcus gattii* and *C. neoformans in vitro*, by assessing the toxicity of the compounds showing the greatest antifungal activity in VERO cells and murine macrophages. From these results, four compounds were considered promising for further studies because they displayed low cytotoxicity and significant antifungal activity. The heterocyclic compounds **1b**, **1c**, **1d**, and **1m** have antifungal activity levels between that of amphotericin B and fluconazole *in vitro*. The death curve of *Cryptococcus* spp. treated with these four compounds was similar to the curve obtained for amphotericin B, in that we observed a significant reduction in cell viability within the first 24 h of treatment. Additionally, we found that there was no effect when these compounds were combined with amphotericin and fluconazole, except for **1c**, which antagonized the effect of amphotericin B against *C. gattii,* also reflected in the reduction of the post-antifungal effect (PAFE); however, this interaction did not alter the ergosterol content. The results shown in this paper reveal the discovery of novel thiazole compounds, which are easy to synthesize, and with potentially exhibit antifungal activity, and display low cytotoxicity in normal mammalian cells. These compounds can be used as prototypes for the design of new antifungal drugs against *C. gattii* and *C. neoformans*.

Keywords: *Cryptococcus*, antifungal, cytotoxicity**,** thiazole.

INTRODUCTION

Cryptococcosis is an opportunistic or primary fungal infection caused by yeasts of the genus *Cryptococcus*. These encapsulated yeasts can infect many animals including humans, leading to severe clinical conditions such as meningoencephalitis [1]. The species *Cryptococcus neoformans* and *C. gattii* are the main etiological agents of cryptococcosis, which is usually acquired by the inhalation of basidiospores or desiccated environmental yeast [2]. The opportunistic disease, which is associated with immunosuppression, is mainly caused by *C. neoformans*, while the primary infection occurs mainly in immunocompetent hosts infected with *C. gattii* [3, 4].

After infection by the inhalation of infectious seedlings, *Cryptococcus* spp. can progress into a regressive form and develop into extrapulmonary foci, and these foci may be reactivated years after infection [5]. Both the opportunistic and primary forms of cryptococcosis are capable of causing meningoencephalitis with severe and fatal outcomes, and may present no obvious lung lesions, fungemia, or secondary foci in various organs such as the skin, bone, and kidney [6, 7].

Estimates show that one million cases of cryptococcosis occur annually, and mortality rates can reach 60% [8, 6]. In Brazil, cryptococcosis is a fungal disease with the highest mortality rate in seropositive individuals [9]. In sub-Saharan Africa, the mortality rate in individuals with AIDS can exceed 90%. In this region, azoles, especially fluconazole, are used as a primary therapy for cryptococcosis, and amphotericin B and flucytosine are usually not used due to their high cost and the difficulty of administering and monitoring treatment [10]. Thus, fluconazole remains the only therapeutic option for most patients in sub-Saharan Africa, which has led to an increased rate of resistance against fluconazole treatment for *Cryptococcus* spp. and resulted in treatment failures [11, 12]. In Brazil, cutaneous and systemic forms of cryptococcosis treatment are used. The systemic therapeutic regimen of choice is amphotericin B, as deoxycholate or in lipid formulation, for 6 weeks in the acute phase of the disease, followed by fluconazole in the consolidation phase. In pulmonary infections or when symptoms are considered clinically mild, fluconazole or itraconazole

treatment is used for 6 to 12 months [13]. The increased incidence of invasive fungal infections is faced with a limited arsenal of treatment options. In addition, one of the biggest problems has been the increasing emergence of fungal resistance to the antifungal agents. Azoles drugs are the most widely used class in medical practice and since its creation has been to increase the resistance [14]. Di Santo [14] also showed new compounds are being studied with potent activity against *C. neoformans*; as A-192411.29, zofimarin and R-135853. A-192411.29 is a novel compound derived by total synthesis from the structural template of the echinocandins. Zofimarin is a sordarin analogue. The R-135853 is a modification of zofimarin, with antifungal activity against *C. neoformans* including fluconazole-resistant strains.

Although treatments already exist for the fungal infections of clinical interest, the search for new drugs is necessary because the increase emergence of resistance, the number of these available antifungal drugs is limited, and they cause serious and toxic side effects [15]. Thus, there is a real need for new therapeutic options for the treatment of cryptococcosis. With this goal, and based on the wide range of biological activities exhibited by thiazole heterocycles [16, 17, 18], we report here the synthesis and screening of thiazole heterocyclic compounds (**Figure 1** and **2**) with therapeutic potential against cryptococcosis. Some of these molecules have shown promising antifungal activity *in vitro*. To the best of our knowledge, six of the synthesized heterocycles have not been previously published (**1a**, **1c**, **1d**, **1k**, **1l**, and **1m**).

MATERIALS AND METHODS

1. Chemistry

Melting points were determined on a Microchemical MQAPF 301 apparatus and are reported uncorrected. FT-IR spectra were recorded using a Perkin Elmer Spectrum One infrared spectrometer and absorptions are reported as wave numbers (cm^{-1}). All NMR spectra were recorded on a Bruker Avance DPX 200 spectrometer (200 MHz). Chemical shifts are given in d (ppm) scale and *J* values are given in Hz. All reagents of analytical grade were obtained from commercial suppliers and used without previous purification. The thiosemicarbazones (**3a-i**) and their cyclic derivatives (**1a-m**) were synthesized according to

methodology previously described in the literature [17, 18, 19]. The synthesis of compounds **1b** and **1f** [15], **1e** [20], **1g** [21] **1h** [22], **1i** [23], and **1j** [24] was previously reported in the literature. The logarithm of the compounds' partition coefficient (ClogP) was calculated using the ALOGPS 2.1 program (http://www.vcclab.org/lab/alogps/).

(*E*)-2-[(2-benzylidenecyclohexylidene)hydrazinyl]-4-phenylthiazole (**1a**). Obtained as a pale yellow solid, yield 66%; mp 164.2-166.8 ºC. ^{1}H NMR (200 MHz, $CDCl_3$), δ/ppm: 8.71 (1H, s), 7.75 (2H, m), 7.39 (9 H, m), 6.77 (1 H, s), 2.73-2.69 (4H, m), 1.83-1.66 (4H, m); ^{13}C NMR (50 MHz, $CDCl_3$), δ/ppm: 169.62, 158-29, 143.52, 136.50, 134.60, 129-9; 129-83, 129.59, 129.25, 128.19, 127.56, 125.61, 101.75, 28.64 (C-2), 28.37 (C-4), 23.79 (C-6), 22.71 (C-5); HRMS (*m*/*z*) 360.1535 $[M+H]^+$, calcd 360.1529 $C_{22}H_{22}N_3S^+$.

(*E*)-2-[(2-benzylidenecyclohexylidene)hydrazinyl]-4-(chlorophenyl)thiazole (**1k**). Obtained as a pale yellow solid, yield 95%; mp 180.1-181.4 ºC; ^{1}H NMR (200 MHz, DMSO-d6), δ/ppm: 7.85 (2H, d, *J* = 8.0 Hz), 7.45 (2H, d; *J* = 8,0 Hz), 7.35 (6H, m); 7.05 (1H, s), 2.65 (4H, m), 1.67-1.56 (4H, m); ^{13}C NMR (50 MHz, DMSO-d6), δ/ppm: 170.07, 136.69, 135.85, 132.10, 129.66, 128.70, 128.36, 127.30, 126.81, 104.96, 28.54, 27.54, 23.72, 22.50; HRMS (*m*/*z*) 394.1142 $[M+H]^+$, calcd 394.1139 $C_{22}H_{21}ClN_3S^+$.

(*E*)-2-[(2-benzylidenecyclohexylidene)hydrazinyl]-4-(methoxyphenyl)thiazole (**1l**). Obtained as a pale yellow solid, yield 79%; mp 155.9-156.9 ºC; ^{1}H NMR (200 MHz, $CDCl_3$), δ/ppm: 8.33 (1H, s), 7.69 (2H, m), 7.39-7.21 (6 H, m), 6.98 (2 H, m), 6.62 (1H, s), 3.83 (3H, s), 2.74-2.69 (4H, m), 1.83-1.66 (4H, m); ^{13}C NMR (50 MHz, $CDCl_3$), δ/ppm: 169.48, 160.55, 158.03, 143.34, 136.53, 134.62, 129.83, 128.18, 127,5 (C-4''); 127.08, 114.61, 99.67, 55.34, 28.65, 28.28, 23.78, 22.70.

(*E*)-3-[2-(4-(4-methoxyphenyl)thiazol-2-yl)hydrazono)]butan-1-ol (**1c**). Obtained as a pale yellow solid, yield 88%; mp 189.2-190.4 ºC; ^{1}H NMR (200 MHz, DMSO-d6), δ/ppm: 7.71 (2H, d, *J* = 8.0 Hz), 7.17 (1H, s), 7.01 (2H, d, *J* = 8.0 Hz), 6.11 (1H, s), 3.78 (3H, s), 3.65 (2H, t, *J* = 8.0 Hz), 2.47 (2H, d *J* = 8.0 Hz), 2.05 (3H, s); ^{13}C NMR (50 MHz, DMSO-d6), δ/ppm: 169.59, 159.56, 157.12, 144.52, 127.32, 124.14, 114.24, 102.05, 55.22, 55.32, 41.38, 17.17; HRMS (*m*/*z*) 392.1119 $[M+H]^+$, calcd 392.1114 $C_{14}H_{18}N_3O_2S^+$.

(*E*)-2-[2-(cyclohexylmethylene)hydrazinyl)]-4-phenylthiazole (**1d**). Obtained as a pale yellow solid, yield 76%; mp 138.3 – 140.3 ºC; HRMS (*m*/*z*) 286.1372 $[M+H]^+$, calcd 286.1372 $C_{16}H_{20}N_3S^+$.

(*E*)-2-[2-(cyclohexylmethylene)hydrazinyl)]-4-)4-methoxyphenyl)thiazole (**1m**). Obtained as a pale yellow solid, yield 96%; mp 146.3 – 148.9 ºC; ^{1}H NMR (200 MHz, DMSO-d6), δ/ppm: 7.74 (2H, d, *J* = 8.0 Hz), 7.26 (1H, d, *J* = 6.0 Hz), 7.00 (1H, s), 6.96 (2H, d, *J* = 8.0 Hz), 3.76 (3H, s), 2.20 (1H, broad s), 1.73-1.66 (5H, m), 1.27-1.21 (5H, m); ^{13}C NMR (50 MHz, DMSO-d6), δ/ppm: 168.58, 158.73, 149.95, 149.43, 127.59, 126.82, 113.89, 100.77, 55.07, 39.92, 29.85, 25.56, 25.00.

2. *In vitro* cytotoxicity assay

2.1. Cell lines

The VERO (African green monkey kidney cells) lineage was used as a model of normal cells and was kindly provided by Dr. Erna Kroon (Universidade Federal de Minas Gerais, UFMG). This lineage was maintained in the logarithmic phase of growth in D-MEM supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin enriched with 5% fetal bovine serum. VERO cells were maintained at 37°C in a humidified incubator with 5% CO_2 and 95% air. The medium was changed twice weekly, and the cells were regularly examined and used until 20 passages.

2.2. Peritoneal macrophage isolation and cell culture

Male C57BL/6 mice (20–22g) were obtained from the Biotery Center (CEBIO) of the Institute of Biological Sciences, UFMG. Animal experiments were conducted in accordance with current ethical regulations on animal research (221/2013). Mice were randomized and housed in plastic cages in groups of five per cage. The animals were maintained under standard laboratory conditions at a temperature of 25 ± 2ºC and a photoperiod of 12 h, and received standard mouse chow and water *ad libitum*.

Peritoneal macrophages were obtained from 6 to 8-week-old C57BL/6 mice. Mice were intraperitoneally injected with 2 mL of 3% sodium thioglycollate (Amresco, Solon, OH, USA). After 72 h, mice were euthanized and dipped in 70°GL alcohol for disinfection. The peritoneal membrane was exposed and 3 mL of ice-cold PBS was injected into the peritoneal cavity to collect macrophages. Peritoneal lavage

was obtained and centrifuged (10 min, 150 × *g*, 4°C). The cell pellet was suspended in RPMI (Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (Cultilab, Campinas, Brazil), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma, St. Louis, USA), and cell density was determined by counting in a Neubauer chamber. The macrophages of each mouse were seeded (1 x 10^5 cells/well) in 96-well plates and incubated for 12 h at 37°C in an incubator with 5% CO_2.

2.3. Evaluation of the cytotoxic effects against VERO cells and murine macrophages

VERO cells at 1 x 10^4 cells per well and murine macrophages at 1 x 10^5 cells/well were pre-incubated in 96-well plates for 24 h at 37°C to allow cells to adapt prior to the addition of the test compounds. All experimental compounds and amphotericin B (Sigma, St Louis, USA) were dissolved in DMSO prior to dilution, with the exception of fluconazole (Sigma, St Louis, USA), which was dissolved in sterile deionized water. The half-maximal inhibitory concentration (IC_{50}) was determined over a range of concentrations (eight nonserial dilutions: from 100 to 0.0128 μM). VERO cells and peritoneal macrophages were incubated with the compounds or 0.5% DMSO as a control in a 5% CO_2/95% air-humidified atmosphere at 37ºC for 48 h. Cell viability was estimated by measuring the rate of the mitochondrial reduction of tetrazolium–dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Amresco, Solon, USA).

Controls included drug-containing medium (background) and drug-free complete medium. Drug-free complete medium was used as a control (blank) and was treated in the same way as the drug-containing media. Results were expressed as a percentage of inhibition of cell viability compared to the 0.5% DMSO control and were calculated as follows: % inhibition of cell viability (%) = 100 - (mean OD treated – mean OD background)/(mean OD untreated culture, i.e. 0.5% DMSO – mean OD blank wells) x 100. Interactions between compounds and media were estimated on the basis of variations between drug-containing media and drug-free media to avoid false-positives or false-negatives [27]. All samples were tested in triplicate in two independent experiments. The cytotoxicity of fluconazole and amphotericin B was evaluated under the same experimental conditions as the positive controls.

2.4. Selectivity index determination

The IC_{50} and minimum inhibitory concentration (MIC) data were used to calculate the selectivity index (SI) of each compound as was done previously by Protopopova et al. [28]. The SI was determined by the ratio of the IC_{50} value in VERO cells or murine macrophages to the IC_{50} value in MIC and calculated as $SI = IC_{50}/MIC$.

2.5. Statistical analysis

The IC_{50} value for cytotoxicity activity was determined using Prism 5.0® (GraphPad Software Inc.). Data were presented as median and 95% confidence interval. Statistical differences between the treatments and the control were evaluated by an analysis of variance (ANOVA) test. Statistical differences between the treatments and the control were evaluated by an ANOVA test followed by a Bonferroni test ($P > 0.05$).

3. Determining the antifungal activity

3.1. Fungal strains and inoculum quantification

Cryptococcus gattii (ATCC24065, ATCC32608) and *C. neoformans* (ATCC24067, ATCC28957, ATCC62066) were obtained from the Culture Collection of the University of Georgia (Atlanta, GA, USA). Ten isolates of *C. gattii* (L135/03, L28/02, 23/10993, 196L/03, 1913 ER, L27/01, LMM 818, L24/01 - clinical isolates; 547/OTTI/94-PI-10, ICB 181 - environmental isolates), and nine clinical isolates of *C. neoformans* (96806, 5396, LMM820, F10, 28JF, VM-MCMMPI, 27JF, WP – clinical isolates; CN31 - environmental isolate), all from the culture collection of the Mycology Laboratory/Institute of Biological Sciences - UFMG, were used in this study. All yeast strains were stored frozen at -80ºC.

Starting inoculates of *Cryptococcus* isolates were cultured onto Sabouraud dextrose agar (SDA, Himedia, Mumbai, India) twice for 48 h at 35°C to ensure purity and viability. The inoculum was prepared by picking distinct colonies that were suspended in 5 mL of sterile saline (0.85% NaCl), and the transmittance was adjusted to 70% in the wavelength of 530 nm. Therefore, dilution was carried out in RPMI medium supplemented with L-glutamine and buffered to pH 7.0 with 0.165 M morpholine propanesulfonic acid (MOPS) (Sigma, St Louis, USA), adjusting the concentration of the yeast to 1-5 $x10^3$ cells/mL, in accordance with M-27-A3 [29].

3.2. Determination of minimum inhibitory and fungicidal concentration

Broth microdilution testing was performed in accordance with the guidelines in the CLSI document M27-A3 [29]. Susceptibility was determined by the microbroth dilution method, which was performed in sterile flat-bottom 96-well microplates (Difco Laboratories, Detroit, MI, USA). The compounds were dissolved in DMSO (Vetec, RJ, Brazil) and diluted in synthetic RPMI medium. Later, serial dilutions were performed in RPMI. The experimental compounds were tested at concentrations of 0.1 to 62.5 µM. As growth and sterility controls, RPMI alone was used, without the addition of the compounds and solvent. Fluconazole (0.97 to 125 µM) and amphotericin B (0.05 to 7.8 µM) were included as the positive antifungal controls. The plates were incubated at 35°C for 48-72 h. The endpoints were determined visually by comparison with the endpoints of the drug-free growth-control wells. The value of the minimum inhibitory concentration (MIC) was defined as the lowest compound concentration (µM) at which the well was optically clear. The minimal fungicidal concentration (MFC) of each compound tested was determined as described by Espinel-Ingroff [30].

3.3. Time-kill curve procedures

The time-kill curve was determined for compounds against *C. gattii* and *C. neoformans* isolates. In this experiment, the required time of contact between fungi and antifungal drug necessary to kill 100% of the yeast was determined. Microplates were prepared in accordance with the methodology described for the MIC assay containing the experimental substances and the antifungal control (amphotericin B and fluconazole) only the concentrations used were equal to the MIC and twice the MIC in the RPMI medium. The plates were then incubated at 35°C for 0, 6, 12, 24, 48, or 72 h. In sequence, we added MTT (5.0 mg/mL) to determine the reduction in the metabolic cell activity. The plates were incubated at 35°C for 3 h, after which isopropanol was added before the spectrophotometric reading was carried out at 490 nm. Culture controls without antifungal drug were subjected to the same procedures at the same intervals. The results were

confirmed by plating an amount of sample from each well on SDA for colony counting [31]. All kill curves were performed in triplicate.

3.4. Checkerboard microtiter test

Eight serial two-fold dilutions of experimental compounds (0.11 to 15.6 μM) and amphotericin B (0.025 to 3.9 μM) were prepared with the same solvent (DMSO) and dilutions of fluconazole (0.48 to 62.5 μM) were prepared with water as in the MIC test. The checkerboard was prepared in a microtiter plate for multiple combinations of two antimicrobial agents. Each row (x-axis) in the plate contained the same diluted concentration as the first antimicrobial compound, while the concentration in each subsequent row was half of this value. Similarly, each column (y-axis) in the plate contained the same diluted concentration as the second antimicrobial compound, while the concentration in each subsequent column was half of this value. The concentration of the drug combination at which growth was completely inhibited was taken as the effective MIC for the combination. One hundred microliters of inoculate suspension was added to each well and cultured for 48 to 72 h. Fractional inhibitory concentrations (FICs) were calculated as the MIC of the combination of drugs divided by the MIC of drug alone. The same calculation was performed for all experimental compounds. The FIC index (FICI) was calculated by adding both FICs, and this was interpreted in the following manner: an effect was classified as synergistic when its value was ≤0.5, as no interaction when the value fell within the 0.5–4.0 range, and as antagonistic when its value was >4.0 [32].

3.5. Ergosterol quantification

Ergosterol quantification in the fungal cell membrane was performed as described previously by Arthington-Skaggs et al. [33] with modifications. We selected the L27/01 and 196/03 isolates of *C. gattii* as representatives of antagonistic interactions observed between amphotericin B and compound **1c**. Approximately 25 mg of the fungal cell mass was transferred to polypropylene tubes. The yeasts were then treated with **1c**, amphotericin B, and fluconazole at concentrations equal to the MIC and with a combination of amphotericin B and **1c** (both at MIC) in YPD medium for 24 h under agitation at 37°C. A growth control assay was also performed. After incubation, the tubes were centrifuged (Jouan, model BR4i) at 7000 rpm for

10 min at 4°C, and the supernatant was removed. The cells were washed with sterile distilled water. The wet weight of the cell pellet was determined. For the extraction of lipids, 3 mL ethanolic solution of 25% potassium hydroxide was added to each cell mass, followed by agitation for 1 min. The tubes were incubated in a water bath at 85°C for 1 h and then cooled at room temperature. A mixture of 1 mL sterile water and 3 mL n-heptane was added, followed by agitation in a vortex for 3 min. The supernatant was removed, and the reading was performed using a spectrophotometer (Hitachi U-1100) at 282 and 230 nm. A calibration curve with standard ergosterol (Sigma-Aldrich) was constructed and used to calculate the amount of ergosterol. The ergosterol levels were expressed as a percentage of the ergosterol in the growth control.

3.6. Post-antifungal effect

The post-antifungal effect (PAFE) was determined for compound **1c** in combination with amphotericin B for isolate L27/01 of *C. gattii* and isolate CN31 of *C. neoformans*, which showed antagonistic effect when treated with these combinations of drugs. We prepared 96-well plates in according to the methodology described in the Checkerboard microtiter test (item 3.4) with inoculum of the 10^3 cells/mL. The concentrations tested for the drugs alone and in combination against *C. gattii* were 0.11 µM for amphotericin B and 0.45 µM for **1c**. For *C. neoformans* the concentrations were 0.05 µM for amphotericin B and 0.9 µM for **1c**. A growth control with 10^4 cells/mL was performed for the representative increase of 1 $\log_{10}$ of growth for each strain of the *Cryptococcus* spp.. The plates were incubated at 35°C for 1 h. After incubation, the plates were centrifuged (Jouan, model BR4i) at 4000 rpm at 4°C for 30 minutes. The supernatant was removed from each well, and the plates were washed twice with RPMI 1640 medium. A volume of 100 µL was added and the plates were incubated at 35°C for 24 h. At different times, we added MTT (5.0 mg/mL) to determine the reduction in the metabolic cell activity. The plates were incubated at 35°C for 3 h, after which isopropanol was added before the spectrophotometric reading that was carried out at 490 nm. The PAFE was calculated as the time that each strain took to achieve the cell population represented by the control wells (10^4 cells/mL), which is equivalent to an increase of 1 log10 in the yeast number [31].

RESULTS

Synthesis

Initially, 2-benzylidenecyclohexanone **2** was synthesized by an aldol reaction between benzaldehyde and cyclohexanone, producing a 67% yield. Subsequently, the appropriate aldehyde or ketone, including **2**, were subjected to reaction with thiosemicarbazide to give the thiosemicarbazones **3a**-**i** (51-97% yield) [19, 20], which were converted into the corresponding thiazole derivatives **1a**-**m** (66-98% yield), according to the procedure outlined by Dimmock et al. [21] (**Scheme 1**). To correlate lipophilicity with antifungal activity, the logarithm of the compounds' partition coefficient (ClogP) was calculated, and the values obtained ranged from 2.51 to 6.91.

In vitro activity

Thirteen thiazole heterocyclic compounds were initially assayed for their MICs against *C. neoformans* (ATCC24067) and *C. gattii* (ATCC24065). In this first test, **1e**, **1f**, **1g**, **1i**, and **1j** showed MIC values >250 μM (**Table 1**). This value was much higher than that of the other compounds tested, which had MICs from 0.45 to 15.6 μM, which were values considered promising because they resemble the MICs of amphotericin B and fluconazole. From these results, toxicity against VERO cells and murine macrophages was evaluated only for **1a**, **1b**, **1c**, **1d**, **1k**, **1l**, and **1m** (**Table 2**), which were the compounds that showed the best MIC values in this study. These compounds did not affect the viability of VERO cells and murine macrophages at concentrations up to 100 μM, which is reflected by the IC_{50} values of greater than 100 μM. The only difference between the results of the two cytotoxicity assays was with amphotericin B, which in an assay with macrophages showed an IC_{50} value of 53.37 ± 18.46 μM, although VERO cell toxicity was not detected. Considering the MIC values for *C. neoformans* (ATTCC24067) and *C. gattii* (ATCC24065) and the toxicity results for VERO cells and murine macrophages, the SI was calculated. Compounds **1b**, **1c**, **1d**, and **1m** showed the highest SI values ($\geq$ 26), and, therefore, were selected for follow-up tests.

Antifungal activity against twelve strains of *C. gattii* showed MIC values ranging from 1 to 3.4 μM for compound **1b**, 0.9 to 1.7 μM for **1c**, 0.7 to 2.2 μM for **1d**, and 1.4 to 4.5 μM for **1m**. These MIC values were smaller than that observed for fluconazole, (7.8 to 41.7 μM). Amphotericin B exhibited MIC values ranging from 0.04 to 0.14 μM (**Table 3**). MIC values of compounds against strains of *C. neoformans* ranged from 1.2 to 3.5 μM for **1b**, 0.5 to 1.2 μM for **1c**, 1.2 to 2.4 μM for **1d**, and 1.9 to 3.5 μM for **1m**. Concerning amphotericin B and fluconazole, MIC values were 0.04 to 0.14 μM and 3.2 to 31.2 μM, respectively. However, based on the Newman-Keuls multiple comparison test, the MIC_{50} and MIC_{90} values of compounds did not differ significantly from amphotericin B. In contrast, there was a statistically significant difference between the experimental compounds and fluconazole. The same was true for both species of *Cryptococcus*.

The activity of thiazole compounds was reflected in the death curve constructed for the average of all isolates of *C. gattii* and *C. neoformans* tested (**Figure 3**). Compounds **1c** and **1d** and amphotericin B showed about a 90% reduction in viable cells of *C. gattii* over 72 h, while this reduction ranged from 80-90% for compounds **1b** and **1m** and fluconazole in the same period. Curves constructed for *C. neoformans* had similar kinetic profiles between compounds and control drugs.

The average FICI for **1b**, **1c**, **1d**, and **1m** in combination with fluconazole showed values of interaction indifferent to antagonism, yet the geometric mean for the twelve isolates demonstrated the absence of an interaction (**Table 4a** and **b**). The combination with amphotericin B obtained similar results as the combination with fluconazole, with the exception of **1c** against *C. gattii*, which exhibited a geometric mean of 4.29, which indicated antagonism (**Table 5a** and **b**). Ergosterol quantification was performed to evaluate the influence of ergosterol content on the interaction between **1c** and amphotericin B. We observed that only the fluconazole treatment promoted a reduction in ergosterol content, while treatment with amphotericin B and **1c** alone or in combination did not significantly change the amount of ergosterol present in *C. gattii* cells (**Figure 4**).

The PAFE found to *C. gattii* for combination between 1c and amphotericin B was equal to 2.15 h, while for drugs alone was 2.65 h (amphotericin B) and 1.7 h (1c). The PAFE found for *C. neoformans* was

2.1 h for the combination, 2.75 h (amphotericin B) and 2.8 h (1c). In both species the antagonism between compound 1c and amphotericin B reflected in decreased of the PAFE when compared to the effect of these drugs alone (**Table 6**).

DISCUSSION

Fungal infections are a major clinical problem and, compared to bacterial infections, limited therapeutic agents are available for their treatment, among which only some are able to identify unique targets that are not shared with human hosts [34]. Moreover, currently several cases of resistance to current antifungals have been described [35, 13, 36].

The present work evaluated seven compounds previously described in the literature and six unpublished compounds, of which three (**1c**, **1d** and **1m**) were found to be promising for the study of cryptococcosis in an animal model. Of the seven known compounds, only **1b** showed no cytotoxicity and promising antifungal activity against *C. gattii* and *C. neoformans*. Furthermore, Barthi et al. [18] had already reported the antifungal activity of **1b** against *Candida albicans, C. neoformans,* and *Aspergillus flavus*.

Our group has focused on the discovery of fungicidal compounds with low toxicity. In this study, we used VERO cells and murine macrophages as models of normal mammalian cells to evaluate the toxicity of new compounds, and found that the compounds we tested were selective for cells of *Cryptococcus* spp., with less toxicity against the mammalian cells used in this study. Compounds with high selectivity offer the potential for safer therapy with fewer side effects and a mechanism to identify candidates for efficacy studies in mice [37]. There are several reports in the literature that have used these cell types for assessing cytotoxicity in the process of drug development [28, 37, 39, 40]. Magalhães et al. [37] tested new active hydroxyaldimines against VERO cells and found a low toxicity and high selectivity of these compounds; hydroxyaldimine 3A8 was more selective to *Cryptococcus* spp. than to VERO cells. From these findings, the authors considered these compounds good candidates for *in vivo* studies.

We present four thiazole heterocyclic compounds (**1b**, **1c**, **1d** and **1m)** that exhibited especially excellent activity against *Cryptococcus* spp. and which showed no toxic effects in mammalian cells. These

compounds have pharmaceutically acceptable compositions and are easily synthesized from commercially available starting materials with good yields.

In the present study, we observed that the thiazole compounds were able to inhibit and kill the yeast, showing promising activity *in vitro* against both *C. gattii* and *C. neoformans*. The death curves obtained for these compounds were comparable to that observed for the drugs amphotericin B and fluconazole. It is also interesting to note that there was a good correlation between the antifungal activity and ClogP. Ideally, a good lead compound must have a ClogP of less than 5 for a drug to passively cross biological membranes [38]. The most promising compounds **1b** (ClogP = 3.88), **1c** (ClogP = 2.51), **1d**, and **1 m** (ClogP = 5.24) showed ClogP within or very close to this range (Table 2).

When testing the *in vitro* interactions between amphotericin B and fluconazole, we found that the average CIFI values were indifferent to the combination or showed antagonism depending on the strain tested; specifically, **1c** with amphotericin B showed that the average CIFI of 11 isolates of *C. gattii* were equal to 4.29 μM, which is considered an antagonistic interaction. Some studies suggest that antagonism between two or more drugs may occur because they compete for the same target, while no observable interaction indicates that the drugs in combination have no adverse effect on the therapeutic response [41, 42]. Santos et al. [31] evaluated whether an interaction occurred between amphotericin B and fluconazole against various isolates of *C. gattii* and noted the existence of an interaction between these drugs ranging from synergism to antagonism, depending on the isolate and concentration tested. These authors also showed that the antagonistic effects of these drugs could be related to the reduction of ergosterol by fluconazole, leading to lower activity of amphotericin B when used in combination with fluconazole.

In the present study, the incidence of antagonism between the compound **1c** and amphotericin B could not be related to the reduced content of ergosterol in the fungal membrane, which was observed only in cells treated with fluconazole. Generally, azoles interfere with the ergosterol biosynthesis pathway resulting in less extracted ergosterol. In cells treated with **1c** alone as well as in cells treated with the combination of amphotericin B and **1c**, no change in ergosterol biosynthesis was observed, which excludes the possibility

that the antagonism observed was a result of the action of ergosterol in the fungal membrane, culminating in the reduced action of amphotericin B.

The PAFE is a drug-related factor important for determination of the *in vivo* efficacy of antifungal compounds. The PAFE refers to the suppression of fungal growth that persists following brief exposure of organisms to an antifungal drug and subsequent removal of the drug [43]. The PAFE found for amphotericin B is in agreement with the results of Santos et al. [31], who observed PAFE values from 1 to 3 h, with average PAFE equal to 2.1 h.

CONCLUSION

In this study, we investigated thiazole heterocyclic compounds with potential antifungal activity against *Cryptococcus* spp. that are easily synthesized and have low toxicity in mammalian cells. Thus, we present important results primarily regarding the unpublished compounds **1c**, **1d**, and **1m** and the known compound **1b** as promising candidates for *in vivo* studies concerning the treatment of cryptococcosis.

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Conflicts of interest

All authors: None to declare.

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TABLE 1. Screening with 13 thiazole heterocyclic compounds to determine the minimum inhibitory concentration (MIC) in μM against *Cryptococcus neoformans* (ATCC24067) and *Cryptococcus gattii* (ATCC24065).

Compounds	*C. neoformans* (ATCC24067)	*C. gattii* (ATCC24065)
	μM	μM
1a	15.6	15.6
1b	1.9	1.9
1c	0.45	0.9
1d	0.9	0.9
1e	>250	>250
1f	>250	>250
1g	>250	>250
1h	15.6	15.6
1i	>250	>250
1j	>250	>250
1k	7.8	7.8
1l	7.8	7.8
1m	1.9	3.9
Amphotericin B	0.486	0.486

TABLE 2. Cytotoxicity (IC_{50}), selectivity index (SI), and ClogP in VERO cells and murine macrophages.

	IC_{50}VERO	SI	IC_{50} Macrophage	SI	CLogP
	µM		µM		
1a	>100	6.41	>100	6.41	6.31*
1b	>100	29	>100	29	3.88*
1c	>100	111 [a] // 222 [b]	>100	222	2.51*
1d	>100	111	>100	111	5.24*
1h	>100	6.41	>100	6.41	5.25*
1k	>100	12.8	>100	12.8	6.91*
1l	>100	12.8	>100	12.8	6.47*
1m	>100	52.63[a] // 25.64[b]	>100	52.63[a] // 25.64[b]	5.24*
Fluconazole	>100	15.74[a] // 7.87[b]	>100	15.74[a] // 7.87[b]	0.58**
Amphotericin B	>100	205.76	53.37±18.46	109.81	-0.66**

(*) Clog P values were calculated using the ALOGPS 2.1 program (http://www.vcclab.org/lab/alogps/), (**) ClogP values were calculated using the XlogP3 program. http://www.vcclab.org/lab/alogps/ (programa ALOGPS 2.1). SI, selectivity index (SI = IC50/MIC). ([a]), *Cryptococcus neoformans* (ATCC24067). ([b]), *Cryptococcus gattii* (ATCC24065).

TABLE 3. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) against twelve *Cryptococcus gattii* strains and twelve *C. neoformans* strains.

Isolates of *C. gattii*	1c		1d		1b		1m		Amphotericin B		Fluconazole	
	MIC (µM)	MFC (µM)	MIC (µM)	MFC (µM)	MIC (µM)	MFC (µM)	MIC (µM)	MFC (µM)	MIC (µM)	MFC (µM)	MIC (µM)	MFC (µM)
ATCC 24065	0.9	1.9	1.5	3.9	1.5	1.9	1.9	3.9	0.07	0.2	13.0	31.3
ATCC 32608	1.4	3.9	1.4	1.9	2.2	3.9	2.9	3.9	0.14	0.9	13.0	62.5
23/10993	1.4	3.9	1.9	7.8	2.9	3.9	2.6	3.9	0.08	0.1	13.0	>62.5
L28/02	1.4	3.9	1.9	7.8	2.7	3.9	4.2	7.8	0.08	0.1	26.0	>62.5
547/OTTI-97-PI-10	1.7	3.9	1.9	7.8	1.9	7.8	2.6	3.9	0.04	0.1	13.0	>62.5
135L/03	0.9	3.9	1.9	3.9	3.1	3.9	2.6	3.9	0.14	0.5	7.8	31.3
196L/03	1.4	3.9	1.4	3.9	2.2	7.8	2.9	3.9	0.04	0.2	26.0	>62.5
L27/01	0.8	1.9	1.0	1.9	1.5	1.9	2.2	3.9	0.08	0.1	41.7	>62.5
ICB181	0.7	1.9	0.7	3.9	1.0	1.9	1.4	1.9	0.12	0.9	10.4	62.5
L24/01	1.4	3.9	1.4	3.9	1.7	7.8	2.9	3.9	0.04	0.1	13.0	>62.5
LMM 818	1.4	3.9	2.2	3.9	3.4	3.9	4.5	7.8	0.14	0.2	20.8	>62.5
1913 ER	0.9	1.9	1.4	7.8	1.0	7.8	2.2	3.9	0.08	0.1	13.0	>62.5
MIC_{50} / / MFC_{50}	1.4	3.9	1.4	3.9	1.7	3.9	2.6	3.9	0.08	0.2	13	>62.5
MIC_{90} / / MFC_{90}	1.4	3.9	1.9	7.8	3.1	7.8	4.2	7.8	0.14	0.9	26	>62.5

Isolates of *C. neoformans*	1c		1d		1b		1m		Amphotericin		Fluconazole	
	MIC (μM)	MFC (μM)	MIC (μM)	MFC (μM)	MIC (μM)	MFC (μM)	MIC (μM)	MFC (μM)	MIC (μM)	MFC (μM)	MIC (μM)	MFC (μM)
ATCC 24067	0.6	3.9	1.4	3.9	3.5	7.8	3.5	7.8	0.09	0.22	7.8	62.5
ATCC 28957	0.6	3.9	1.2	7.8	2.2	7.8	2.2	3.9	0.04	0.22	3.2	62.5
ATCC 62066	0.6	15.6	1.2	7.8	1.2	3.9	1.9	7.8	0.03	0.22	13.0	>62.5
CN31	0.5	7.8	1.4	1.9	1.9	1.9	1.9	3.9	0.08	0.45	6.5	31.25
96806	0.9	3.9	1.9	3.9	1.9	7.8	1.9	1.9	0.09	0.45	31.2	>62.5
5396	0.5	3.9	1.4	1.9	1.2	0.9	2.2	3.9	0.06	0.11	23.4	62.5
LMM 820	0.5	3.9	1.7	1.9	2.2	3.9	3.2	7.8	0.06	0.22	13.0	62.5
F10	0.7	3.9	1.7	3.9	2.2	7.8	2.2	3.9	0.06	0.11	10.4	>62.5
28JF	0.6	3.9	2.7	7.8	2.6	1.9	3.5	7.8	0.11	0.22	7.8	>62.6
VM-MCMMPI	0.5	3.9	1.4	3.9	1.6	7.8	2.2	3.9	0.07	0.22	11.7	15.6
27JF	1.2	7.8	2.4	7.8	3.2	7.8	3.5	7.8	0.07	0.45	10.4	>62.5
WP	0.9	3.9	2.2	3.9	3.2	3.9	3.2	3.9	0.09	0.22	15.6	>62.5
MIC_{50} / / MFC_{50}	0.6	3.9	1.4	3.9	2.2	3.9	2.2	3.9	0.07	0.22	10.4	>62.5
MIC_{90} / / MFC_{90}	0.9	7.8	2.2	7.8	3.2	7.8	3.5	7.8	0.09	0.45	23.4	>62.5

TABLE 4. Fractional inhibitory concentration index values for compounds 1b, 1d, 1m, and 1c in combination with fluconazole against twelve *Cryptococcus gattii* **(a)** strains and twelve *Cryptococcus neoformans* **(b)** strains. Ind., Indifferent; Ant., Antagonism.

(a)

		1d		1c		1b		1m	
		FICI (µM)	**Interaction**	**FICI (µM)**	**Interaction**	**FICI (µM)**	**Interaction**	**FICI (µM)**	**Interaction**
C. gattii	**ATCC32608**	1.72	Ind.	2.32	Ind.	3.45	Ind.	**4.38**	Ant.
	196L/03	2.47	Ind.	1.95	Ind.	1.84	Ind.	2.28	Ind.
	547/OTTI-97-PI-10	**4.47**	Ant.	3.31	Ind.	**5.96**	Ant.	3.46	Ind.
	135L/03	2.47	Ind.	2.38	Ind.	2.45	Ind.	2.71	Ind.
	L28/02	2.47	Ind.	1.01	Ind.	**4.22**	Ant.	**4.38**	Ant.
	L24/01	2.47	Ind.	3.32	Ind.	**6.46**	Ant.	**6.79**	Ant.
	LMM818	2.47	Ind.	2.32	Ind.	1.59	Ind.	1.19	Ind.
	L27/01	**4.23**	Ant.	2.14	Ind.	1.45	Ind.	1.18	Ind.
	1913ER	2.09	Ind.	2.47	Ind.	2.45	Ind.	2.46	Ind.
	ATCC24065	1.58	Ind.	0.88	Ind.	3.71	Ind.	2.43	Ind.
	23/10993	1.47	Ind.	2.28	Ind.	**4.23**	Ant.	2.46	Ind.
	ICB181	2.58	Ind.	1.99	Ind.	**4.58**	Ant.	**4.46**	Ant.
	Geometric mean	2.41		2.06		3.17		2.82	

(b)

		1d		1c		1b		1m	
		FICI (µM)	**Interaction**	**FICI (µM)**	**Interaction**	**FICI (µM)**	**Interaction**	**FICI (µM)**	**Interaction**
C. neoformans	**28JF**	1.99	Ind.	1.47	Ind.	3.77	Ind.	**4.59**	Ant.
	96806	1.76	Ind.	2.01	Ind.	1.01	Ind.	1.01	Ind.
	ATCC62066	2.04	Ind.	2.3	Ind.	2.46	Ind.	3.58	Ind.
	CN31	3.97	Ind.	**4.38**	Ant.	2.71	Ind.	2.58	Ind.
	WP	2.47	Ind.	2.32	Ind.	2.46	Ind.	2.21	Ind.
	5396	2.4	Ind.	1.32	Ind.	3.46	Ind.	3.48	Ind.
	VM-MCMMPI	**4.47**	Ant.	**4.33**	Ant.	**4.21**	Ant.	**4.46**	Ant.
	27JF	**6.8**	Ant.	**7.44**	Ant.	3.33	Ind.	**4.59**	Ant.
	LMM820	**4.24**	Ant.	2.1	Ind.	**4.46**	Ant.	**4.59**	Ant.
	ATCC28957	**4.47**	Ant.	2.28	Ind.	3.36	Ind.	2.51	Ind.
	ATCC24067	3.59	Ind.	1.92	Ind.	4.46	Ant.	3.97	Ind.
	F10	**4.47**	Ant.	**4.33**	Ant.	1.98	Ind.	2.46	Ind.
	Geometric mean	3.28		2.64		2.93		3.09	

TABLE 5. Fractional inhibitory concentration index values for compounds 1b, 1d, 1m, and 1c in combination with amphotericin B against twelve *Cryptococcus gattii* strains and twelve *Cryptococcus neoformans* strains. Ind., Indifferent; Ant., Antagonism.

		1d		1c		1b		1m	
		FICI (μM)	**Interaction**	**FICI(μM)**	**Interaction**	**FICI (μM)**	**Interaction**	**FICI(μM)**	**Interaction**
C. gattii	**ATCC32608**	2.58	Ind.	3.14	Ind.	1.7	Ind.	2.78	Ind.
	196L/03	3.97	Ind.	**9.38**	Ant.	1.46	Ind.	1.58	Ind.
	547/OTTI-97-PI-10	**4.74**	Ant.	2.98	Ind.	2.66	Ind.	2.58	Ind.
	135L/03	2.67	Ind.	**4.98**	Ant.	1.46	Ind.	1.46	Ind.
	L28/02	1.34	Ind.	2.78	Ind.	2.66	Ind.	2.18	Ind.
	L24/01	**4.87**	Ant.	**4.98**	Ant.	2.16	Ind.	2.38	Ind.
	LMM818	2.58	Ind.	**4.98**	Ant.	2.58	Ind.	1.46	Ind.
	L27/01	1.34	Ind.	**4.61**	Ant.	2.48	Ind.	2.66	Ind.
	1913ER	1.47	Ind.	2.78	Ind.	1.04	Ind.	1.75	Ind.
	ATCC24065	1.72	Ind.	2.98	Ind.	1.2	Ind.	2.00	Ind.
	23/10993	2.67	Ind.	3.51	Ind.	2.18	Ind.	2.36	Ind.
	ICB181	**4.98**	Ant.	**9.55**	Ant.	1.28	Ind.	1.73	Ind.
	Geometric mean	2.61		4.29		1.81		2.03	

		1d		1c		1b		1m	
		FICI (μM)	**Interaction**	**FICI(μM)**	**Interaction**	**FICI (μM)**	**Interaction**	**FICI(μM)**	**Interaction**
C. neoformans	**28JF**	1.38	Ind.	2.58	Ind.	2.46	Ind.	1.75	Ind.
	96806	2.67	Ind.	2.78	Ind.	2.46	Ind.	1.46	Ind.
	ATCC62066	2.47	Ind.	2.58	Ind.	3.06	Ind.	2.58	Ind.
	CN31	2.47	Ind.	**4.98**	Ant.	2.66	Ind.	2.78	Ind.
	WP	2.41	Ind.	2.78	Ind.	1.46	Ind.	1.46	Ind.
	5396	1.15	Ind.	1.58	Ind.	**4.86**	Ant.	2.07	Ind.
	VM-MCMMPI	1.47	Ind.	1.58	Ind.	2.66	Ind.	2.16	Ind.
	27JF	2.58	Ind.	2.67	Ind.	2.66	Ind.	2.78	Ind.
	LMM820	**4.3**	Ant.	2.58	Ind.	2.66	Ind.	2.78	Ind.
	ATCC28957	1.09	Ind.	1.98	Ind.	2.22	Ind.	2.07	Ind.
	ATCC24067	2.67	Ind.	2.78	Ind.	1.46	Ind.	2.36	Ind.
	F10	2.62	Ind.	2.47	Ind.	2.58	Ind.	1.46	Ind.
	Geometric mean	2.11		2.50		2.48		2.08	

TABLE 6. Post-antifungal effect (PAFE) of compound **1c** in combination with amphotericin B (AmB) against *C. gattii* L27/01 and *C. neoformans* CN31 following by 1 hour exposure to antagonistic concentrations of the drugs.

	PAFE (mean ± SD)		
	AmB (0.11µM) + 1c (0.45 µM)	**AmB (0.11µM)**	**1c (0.45 µM)**
C. gattii L27/01	2.15 ± 0.025	2.65 ± 0.15	1.7 ± 0.2
	AmB (0.05µM) + 1c (0.9 µM)	**AmB (0.05µM)**	**1c (0.9 µM)**
C. neoformans CN31	2.1 ± 0.1	2.75 ± 0.45	2.8 ± 0.6

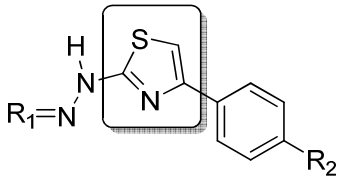


FIGURE 1. General structure of the synthesized thiazole derivatives.

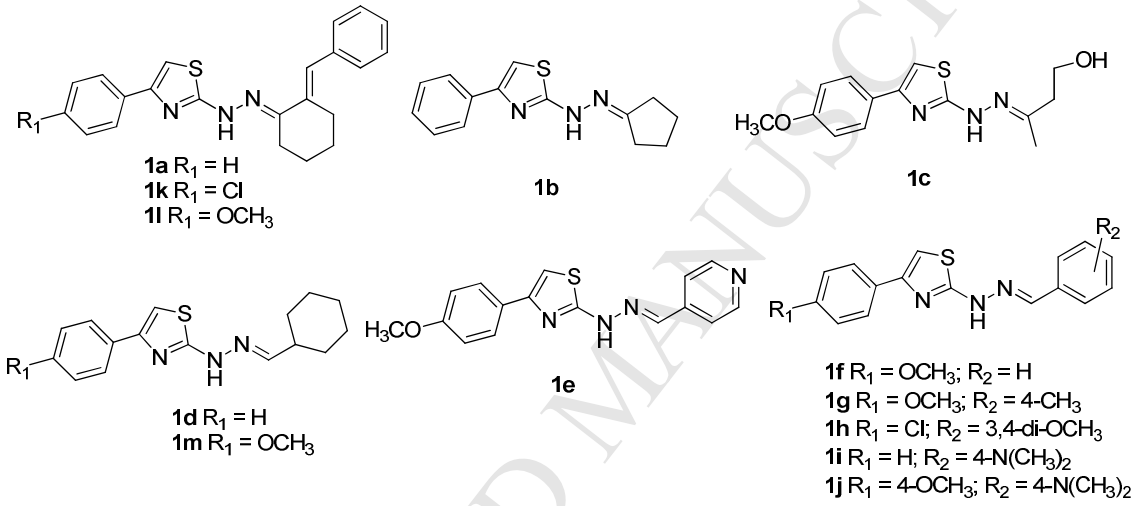
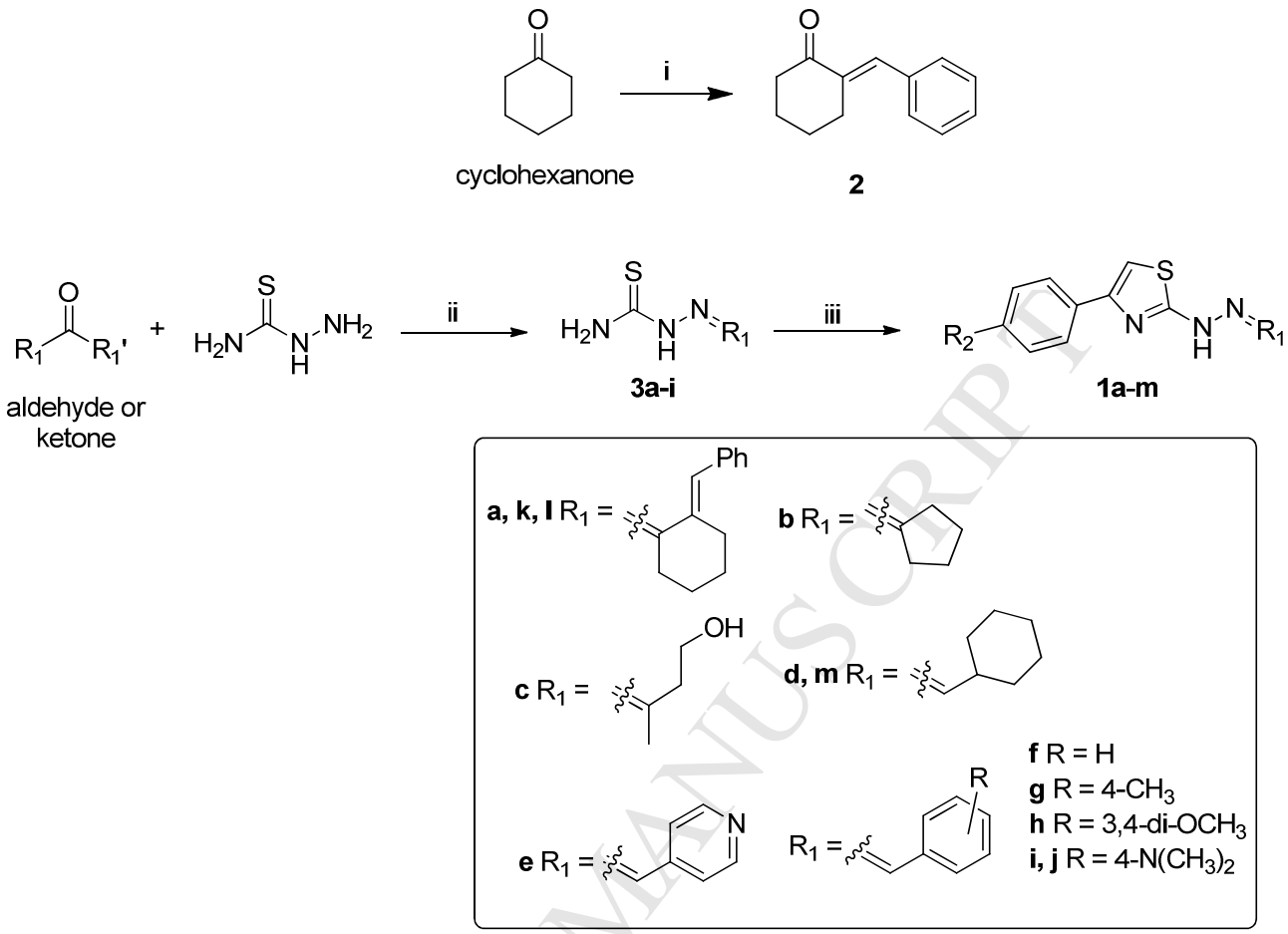


FIGURE 2. Chemical structure of the thiazole derivatives **1a-m**.



SCHEME 1. Reagents and conditions: (i) benzaldehyde, NaOH 1M, reflux, 67% yield; (ii) AcOH, EtOH, reflux, 57-97% yield; (iii) α-bromoacetophenone non-substituted or 4-substituted, *i*-PrOH, reflux, 66-98% yield.

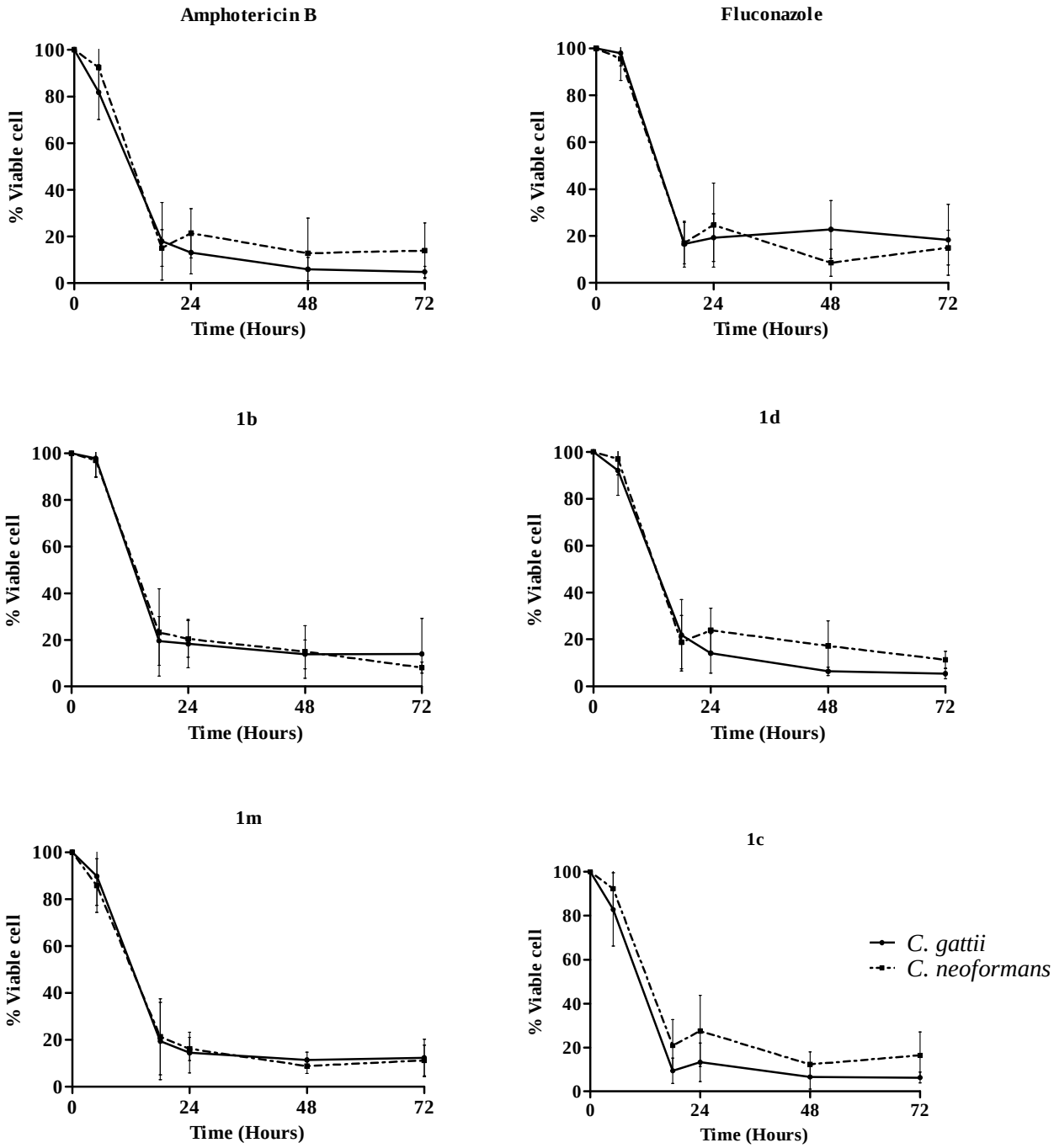


FIGURE 3. Time-kill curves of **1b**, **1d**, **1m**, **1c**, amphotericin B, and fluconazole against twelve *Cryptococcus neoformans* and twelve *Cryptococcus gattii* strains**.** The concentrations against *C. gattii* were **1b** (3.9 μM), 1d (1.9 μM), **1m** (3.9 μM), **1c** (1.9 μM), fluconazole (26 μM) and amphotericin B (0,12 μM). For *C. neoformans* they were 1b (3.9 μM), 1d (1.9 μM), 1m (3.9 μM), 1c (0.9 μM), fluconazole (26 μM), and amphotericin B (0.12 μM).

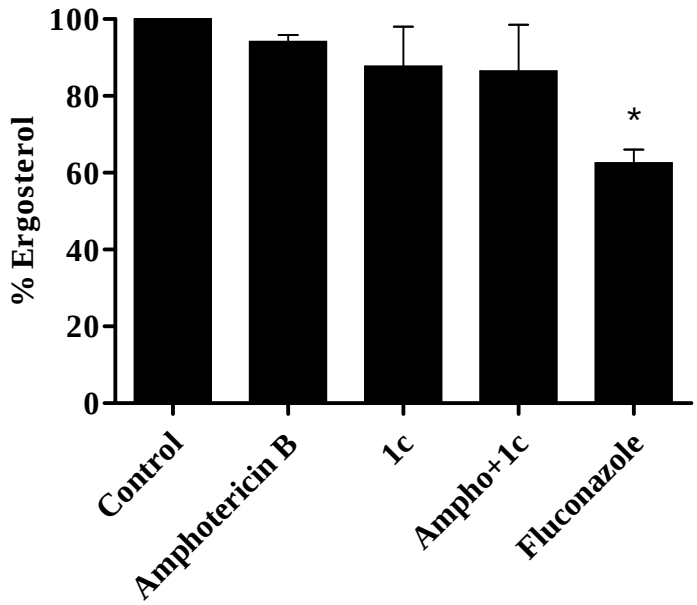


FIGURE 4. Percent ergosterol levels of *Cryptococcus gattii* (L27/01 and 196L/03 isolates) after 24 h of treatment with amphotericin B, **1c** , and fluconazole at MIC, and the combination of amphotericin B with **1c** (both at MIC). *, statistically different ($p<0.05$).

Highlights

We developed some thiazole heterocyclic compounds with promising antifungal activity.

The thiazole displayed low cytotoxicity and significant antifungal activity.

The selected thiazoles can be used as prototypes for the design of new antifungal drugs.